Liquid Chromatographic Determination of Zoalene and Its Metabolites in Chicken Tissues with Electrochemical Detection

A procedure is described for the quantitation of Zoalene (3,5-dinitro-*o*-toluamide) and its 2 major monoamino metabolites in chicken tissues. The method includes blender extraction of tissue with chloroform–ethyl acetate (1 + 1), adsorption of the drug and metabolites on neutral alumina, and subsequent elution of the residues with pH 3.5 formate buffer–methanol (6.5 + 3.5). Recovered residues were separated on a 5 μm C_{18} column with the alumina eluting solvent as the LC mobile phase. The parent drug and metabolites were detected and quantitated with an electrochemical detector in the reductive mode with a minimum level of reliable measurement of 0.1 ppm. Overall mean recoveries greater than 85% were obtained with Zoalene and its 2 monoamino metabolites in breast, thigh, and liver tissues fortified with 0.25–2.00 ppm. The results on tissues from chickens fed a diet containing 0.0125% Zoalene are presented.

Zoalene (Dow Chemical USA brand name for dinitolmide, 3,5-dinitro-*o*-toluamide) is used for the prevention and control of caecal and intestinal coccidiosis in poultry. Although the tolerance levels (1) in poultry tissues are relatively high (liver 6.0 ppm; muscle 3.0 ppm) and include its major metabolite 3-amino-5-nitro-*o*-toluamide, no suitable procedure is available for quantitative residue analysis. The published procedures are time consuming and cumbersome, and include separate steps for the colorimetric determination of the parent drug (2) and metabolite (3). Because of these factors as well as the rapid metabolism of Zoalene, few studies have been conducted to determine the extent of the residue problem in poultry tissues. In a recent publication (4) we described a thin layer chromatographic (TLC) screening procedure for detecting Zoalene and its monoamino metabolites in the presence of other nitro-containing coccidiostats and sulfa drugs. This study, which is an extension of that work, presents a rapid and quantitative liquid chromatographic (LC) procedure, using electrochemical detection in the reductive mode for Zoalene and its monoamino metabolites in chicken tissues.

METHOD

Reagents and Materials

(a) *Solvents.*—Ethyl acetate (EtOAc) and methanol (MeOH) (distilled in glass, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442); chloroform, Baker Analyzed reagent (J. T. Baker Chemical Co., Phillipsburg, NJ 08665).

(b) *Zoalene.*—Salsbury Laboratories, Charles City, IA 50616; 3-amino-5-nitro-*o*-toluamide (3-ANOT)—gift from Dow Chemical USA, Midland, MI 48640; 5-amino-3-nitro-*o*-toluamide (5-ANOT)—see Acknowledgments.

(c) *Tissue homogenizer.*—Brinkmann Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY 11590).

(d) *Centrifuge.*—IEC CENTRA-7R refrigerated centrifuge rotor No. 822A (International Equipment Co., Needham Heights, MA 02194).

(e) *Neutral alumina.*—Brockman Activity I, 80–200 mesh (Fisher Scientific Co., Fairlawn, NJ 07410). Insert 5 mm glass bead into 5 mL pipet tip (Rainin Instrument Co., Woburn, MA 01801). Layer glass bead with 0.5 cm sea sand and 1.25 cm bed of neutral alumina. Pack firmly by gently tapping top of pipet tip. Add 0.25 cm sea sand. Wash column with two 2 mL portions of $CHCl_3$–EtOAc (1 + 1) before use.

(f) *Liquid chromatography.*—Altex Model 100A pump (Altex Scientific Inc., Berkeley, CA 94710) connected to BAS Model LC-45 amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN 47905): glassy carbon electrode—0.85 V vs Ag/AgCl, 50 nA, full scale. Altex Model 210 sampling valve with 50 μL loop. Column: 25 cm × 4.6 mm id 5 μm C_{18} (Alltech Associates, Deerfield, IL 60015). Mobile phase: pH 3.5 formate buffer (0.2 mole formic acid; 0.1 mole KOH; 0.002 mole EDTA per L)–MeOH (6.5 + 3.5) purged with helium. Elute samples isocratically at 0.8 mL/min flow rate. Sampling technique: Purge 2 mL sample in 9 mL vial with helium for 2 min to exclude oxygen from system. Draw ca 1 mL sample through capillary tubing into loop, by way of vent, with hypodermic syringe inserted in sampling valve in "load" position.

Analytical Method

Place 2.5 g frozen, ground tissue into 50 mL polypropylene centrifuge tube. Let sample partially thaw. Add 20 mL $CHCl_3$–EtOAc (1 + 1) and blend 30 s with Polytron homogenizer at low speed. Centrifuge 5 min at 3500 rpm. Remove aqueous layer and discard. Recover solvent and filter through small plug of glass wool packed tightly in 4 mL disposable Pasteur pipet and collect filtrate. Pass 2 mL filtrate through neutral alumina column. Wash sides of column and alumina with 3.25 mL $CHCl_3$ in 0.75, 0.75, 0.75, and 1.0 mL increments. Remove

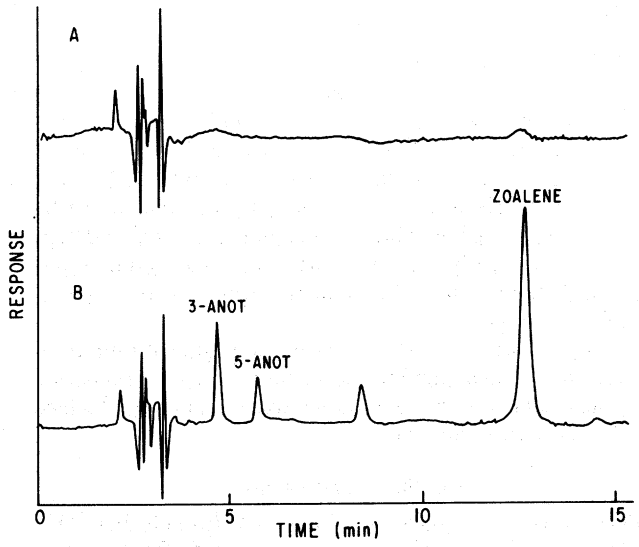

Figure 1. LC chromatograms of (A) extract of control liver and (B) extract of liver from chicken (Table 2, Bird 2) fed a diet containing 0.0125% Zoalene.

Received May 10, 1985. Accepted June 26, 1985.

[1]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1. Percent recovery[a] of Zoalene, 3-ANOT, and 5-ANOT from fortified chicken tissues

Tissue	ppm, added	Recovery, % Zoalene	3-ANOT	5-ANOT
Liver	0.25	99.3 ± 2.7	93.5 ± 2.6	83.5 ± 1.7
	0.50	94.3 ± 2.6	88.8 ± 2.8	80.6 ± 3.9
	1.00	99.5 ± 1.5	92.2 ± 1.3	86.5 ± 3.0
	2.00	99.2 ± 2.2	90.1 ± 2.5	84.6 ± 2.5
Breast	0.25	95.6 ± 2.9	89.1 ± 3.1	83.8 ± 1.2
	0.50	97.7 ± 5.4	85.5 ± 3.0	87.7 ± 3.3
	1.00	97.5 ± 4.5	87.1 ± 6.0	84.9 ± 5.3
	2.00	99.0 ± 2.7	89.5 ± 2.2	85.7 ± 2.0
Thigh	0.25	99.1 ± 3.9	95.7 ± 3.6	86.8 ± 3.7
	0.50	94.4 ± 5.9	89.6 ± 4.9	84.4 ± 3.9
	1.00	98.3 ± 2.4	91.0 ± 1.2	87.2 ± 1.6
	2.00	96.4 ± 2.9	89.3 ± 1.8	87.4 ± 1.8

[a]Mean and standard deviation of 6 determinations at each concentration.

Table 2. Incurred residues in chicken tissues[a]

Bird	Tissue	Concentration, ppm[b] 3-ANOT	5-ANOT	Zoalene
1	Liver	0.19 ± 0.01	0.16 ± 0.02	0.34 ± 0.02
	Breast	0.11 ± 0.01	0.06 ± 0.00	0.26 ± 0.01
	Thigh	0.12 ± 0.01	0.08 ± 0.01	0.21 ± 0.00
2	Liver	0.29 ± 0.02	0.20 ± 0.03	0.67 ± 0.00
	Breast	0.10 ± 0.00	0.06 ± 0.00	0.67 ± 0.02
	Thigh	0.15 ± 0.01	0.14 ± 0.02	0.46 ± 0.01
3	Liver	0.26 ± 0.01	0.18 ± 0.00	0.37 ± 0.02
	Breast	0.12 ± 0.01	0.10 ± 0.00	0.33 ± 0.01
	Thigh	0.15 ± 0.00	0.15 ± 0.01	0.13 ± 0.02

[a]Birds fed a diet containing 0.0125% Zoalene for 10 weeks.
[b]Mean and standard deviation of duplicate samples.

excess $CHCl_3$ from column with air pressure and maintain pressure until column dries as evidenced by disappearance of moisture on outside of column. Elute column with LC mobile phase, collecting first 2 mL effluent in 2 mL volumetric flask. Shake stoppered flask thoroughly and transfer effluent to 9 mL screw-cap specimen vial with disposable Pasteur pipet. Inject 50 μL sample onto LC column according to procedure described above.

Recovery Studies

Spike drug-free frozen, ground tissues by injection at several sites with methanol solutions of drug and metabolites. Keep spiked samples frozen for 1 h before analysis. Use unspiked tissues as controls.

Residues

Liver and muscle tissues were obtained from 10 week old White Leghorn roosters started as 1 day old chicks on feed containing 0.0125% Zoalene. Liver tissues were placed in dry ice within 3 min and muscle tissues within 10 min after sacrificing birds. Tissues were individually blended in Virtis blender after partial thawing, immediately refrozen, and kept in dry ice until analyzed. All tissues were analyzed in duplicate. Birds fed the diet without added drug served as source of control tissues.

Recovery data on fortified tissue and concentrations of drug residues and metabolites were determined by comparing LC peak heights with those of known concentrations of Zoalene and 2 aminonitrotoluamide compounds.

Results and Discussion

Previously it was observed (4) that in addition to 3-ANOT, 5-ANOT and 3 relatively minor, unidentified, Bratton-Marshall (BM)-positive compounds were detected by TLC in extracts of liver tissues obtained from chickens fed a diet containing Zoalene. LC of liver extracts in this study (Figure 1) reveals the presence of 2 unknown metabolites (RT = 8.4 and 14.5 min)—one of which may be of major importance. These same 2 metabolites were not detected in breast and thigh muscle. The relative concentration of the principal unknown metabolite (RT 8.4 min) to 3-ANOT and 5-ANOT in this study, compared to that observed in earlier TLC studies, may be attributed to the difference in time between sacrificing the birds and placing the excised tissues in dry ice. Zoalene is rapidly metabolized (3, 4) in vitro to 3-ANOT and 5-ANOT by liver tissues. We observed no detectable in vitro metabolism in breast or thigh muscle.

In addition to the unknown metabolites referred to above, an unknown polar metabolite, obtained by subjecting livers from Zoalene-fed chickens to the procedure for detecting N^4-glucopyranosylsulfamethazine in swine livers (5), was also observed on TLC plates. The unknown polar metabolite liberated 5-ANOT (tentative identification), but not 3-ANOT, when exposed to HCl fumes before TLC procedures, suggesting that it was a conjugate. Sufficient amount of sample was not available to identify the polar and nonpolar metabolites reported here and therefore they are not included in the recovery or incurred residue studies.

Table 1 summarizes the recovery studies on liver, breast, and thigh muscles each spiked with 0.25, 0.50, 1.0, and 2.0 ppm Zoalene, 3-ANOT, and 5-ANOT. The recovery of the parent drug and monoamino metabolites was relatively uniform among tissues and concentrations. The overall mean recovery for the 3 tissues was 90.1% (CV = 4.38%) for 3-ANOT; 85.3% (CV = 4.04%) for 5-ANOT; and 97.5% (CV = 3.85%) for Zoalene.

Table 2 presents the results of duplicate analyses of tissues obtained from three 10 week old birds started on feed containing 0.0125% Zoalene and continued to the day of sacrifice. The results confirm reproducibility of the analytical procedure. The levels present in tissues of the birds reported here are significantly lower than the tolerance levels set by the U.S. *Code of Federal Regulations* (1) and levels reported by others (2, 3). Wide variations in tissue levels have been attributed (6, 7) to the variability among individual birds, environmental factors, the rapid disappearance of Zoalene and metabolites from tissues when birds are removed from feed, and a decrease in food consumption per unit of body weight as the bird ages. In the latter respect, preliminary studies on liver tissues of five 5 week old birds fed a diet containing 0.0125% Zoalene, with no withdrawal, showed total residues (i.e., Zoalene + metabolites) ranging from 5.9 to 8.9 ppm.

In conclusion, using the procedure described here, an analyst can concurrently prepare 2 tissue extracts for LC every 45 min; LC analyses can be performed at 15–20 min intervals.

Acknowledgments

The authors thank John W. Pensabene (Eastern Regional Research Center) for synthesizing 5-ANOT, and Stanley E. Katz (Rutgers University, Cook College) for arranging the chicken feeding study.

References

(1) *U.S. Code of Federal Regulations* (1981) 21.566.700
(2) Smith, G. N., Thiegs, B. J., & Swank, M. G. (1961) *J. Agric. Food Chem.* **9**, 197–201
(3) Thiegs, B. J., Smith, G. N., & Bevirt, J. L. (1961) *J. Agric. Food Chem.* **9**, 201–204
(4) Parks, O. W. (1985) *J. Assoc. Off. Anal. Chem.* **68**, 20–23
(5) Parks, O. W. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 566–569
(6) Bevirt, J. L., & Thiegs, B. J. (1961) *Poult. Sci.* **40**, 1335–1341
(7) Smith, G. N., Thiegs, B. J., & Ludwig, P. D. (1963) *J. Agric. Food Chem.* **11**, 247–251